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Secretion of insulin, C-peptide, proinsulin, chromogranin A, pancreatic polypeptide, and neuron specific enolase in response to local arterial calcium administration in patients with insulinoma

INAUGURAL-DISSERTATION

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vorgelegt von
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Abstract

Objective: To test whether insulin, C-peptide, proinsulin, chromogranin A (CgA), neuron specific enolase (NSE), and pancreatic polypeptide (PP) are released from insulinoma cells by calcium stimulation during the selective arterial calcium stimulation and hepatic venous sampling test (ASVS).

Design: Prospective case series.

Methods: We determined insulin, C-peptide, proinsulin, CgA, NSE and PP in blood samples obtained during the ASVS test in 19 patients with insulinoma and 6 individuals without insulinoma.

Results: After calcium injection into the artery supplying the insulinoma, a significant 8-fold increase in insulin (range 2.3-117; $p<0.001$), a 3.8-fold increase in C-peptide (range 1.7-32.4; $p<0.001$), a 1.9-fold increase in proinsulin (0.7-5.3, $p<0.001$) and a 1.5-fold increase in PP (range 0.8-4.5; $p=0.017$) was detectable. PP also increased 2.4-fold after injection into the control artery of insulinoma patients (range 0.8-7.9; $p=0.016$) and 2-fold in healthy subjects (0.6-4.8; $p=0.014$). No significant increase of insulin, C-peptide, proinsulin, CgA and NSE concentrations was found after calcium injection into the control artery and in healthy subjects.

Conclusions: Whereas insulin, C-peptide, and proinsulin may be stimulated by calcium injection into an artery supplying the insulinoma, CgA and NSE are not secreted in response to calcium by insulinoma cells. PP may be released by both healthy islet and insulinoma cells after local arterial calcium stimulation. The additional determination of C-peptide (more than 1.5-fold increase) but not proinsulin during the ASVS test may improve diagnostic accuracy.

Introduction

Insulinoma and nesidioblastosis are the most frequent causes of endogenous hyperinsulinemic hypoglycemia in adult patients. Despite continuously improved spatial resolution, radiological methods such as magnetic resonance imaging (MRI), computed tomography (CT) often fail to localize small insulinoma.[1, 2] In patients with nesidioblastosis, these radiological modalities are negative or falsely positive.[3] Selective arterial calcium stimulation and hepatic venous sampling (ASVS) has been established as an accurate method to localize the source of excessive insulin in patients with hyperinsulinemic hypoglycemia.[4-7] We reported earlier that the sensitivity of ASVS in detecting the source of excessive insulin secretion in patients with hyperinsulinemic hypoglycemia is higher (sensitivity 96%) than the sensitivity of CT and/or MRI (sensitivity 59%) or angiography (sensitivity 56%), and even superior to the sensitivity of the intraoperative exploration (sensitivity 88%).[8]

During the ASVS test, calcium is injected into the arteries supplying the pancreas and venous blood is sampled from the right hepatic vein.[4, 5] Since calcium administration elicits a significant increase in insulin secretion by insulinoma but not by normal β-cells, a more than 2-fold rise in insulin levels (determined by "traditional" insulin radioimmunoassays) in the hepatic vein after the injection of calcium into the pancreatic arteries indicates the presence of an insulin secreting tumor in the vascular territory of the artery stimulated.[4, 5] In an earlier study, we reported that the determination of proinsulin during the ASVS test does not improve the diagnostic accuracy of the ASVS test in patients with insulinoma.[9]

The aim of this study was, first, to test whether biochemical markers of neuroendocrine tumors such as chromogranin A (CgA) and neuron specific enolase (NSE) or pancreatic polypeptide (PP)[10] are co-secreted by insulinoma cells in response to calcium stimulation during the ASVS test and second, whether the accuracy of the ASVS test could be improved by the determination of these proteins or by the determination of C-peptide.

Subjects and Methods

Patients

19 consecutive patients with an insulinoma who underwent ASVS for the evaluation of hyperinsulinemic hypoglycemia at the University Hospital of Zurich were included. All patients had a single insulinoma (histologically confirmed) and were cured by surgery. One patient with a glucose-sensitive insulinoma who underwent the ASVS test was excluded because her ASVS was falsely negative and the artery supplying the tumor could not be defined.[11] ASVS was performed in 6 additional patients without evidence for an insulinoma; here called "healthy individuals". Three individuals underwent the angiography for the evaluation of an inactive neuroendocrine tumor (histologically verified; immunhistochemistry for insulin negative) and one patient because of hypoglycemia (in the context of pituitary failure) and suspected pancreatic lesion in the CT scan, which was not confirmed by MRI and clinical follow-up. One patient underwent an ASVS test following successful pancreatic resection of an insulin-secreting tumour because of micrometastasis was found in the peripancreatic adopse tissue.[12] In all patients without evidence for an insulinoma ("healthy individuals"), the absence of an insulin-secreting tumour was supported by a negative 72-h fast. Written informed consent for ASVS was obtained from all patients.

Selective arterial calcium stimulation with hepatic venous sampling (ASVS)

The procedure was performed as previously described.[13] A sampling catheter was placed transfemorally in the right hepatic vein close to its junction with the inferior vena cava. After a standard angiography, the proper hepatic, the gastroduodenal, the splenic, and the superior mesenteric arteries were catheterized. Each artery was stimulated with calcium gluconate (0.025 milliequivalents Ca^{++} per kg body weight). Blood was sampled from the right hepatic vein before (=0) and 30, 60 to 120 seconds after the intraarterial calcium injection. At least 5

minutes were left between each calcium injection. Insulin and C-peptide were determined in all samples. A more than 2-fold rise in insulin within 30-120 seconds after the injection of calcium indicated an insulin secreting tumor in the vascular territory of the artery stimulated (in contrast to no response from normal β-cells). The additional determinations of proinsulin, CgA, NSE, and PP were performed in the samples obtained following calcium stimulation in the artery supplying the tumor (indicated by the maximum increase of insulin concentrations) and a control artery (no increase in insulin concentrations). From each artery, the baseline sample and the sample with the highest insulin concentration following the calcium injection was selected for the additional determinations. From 6 patients without insulinoma, the baseline sample and the sample 60 seconds following calcium injection were selected for the analysis.

Laboratory investigations

Insulin was measured by a solid-phase RIA (CIS Bio international, Oris Industries, Gif-Sur-Yvette, France) with a cross-reactivity of 14% for proinsulin and 0.1% for C-peptide; normal overnight fasting range provided by the manufacturer was 30 to 138 pmol/L; the lower limit of detection 14 pmol/L. C-peptide was determined with a solid-phase radioimmunoassay (CIS Bio international, Oris Industries, Gif-Sur-Yvette, France). The normal overnight fasting range provided by the manufacturer was 350 to 1170 pmol/L; lower limit of detection 120 pmol/L. Cross-reactivity with insulin was below 0.12%, cross-reactivity with proinsulin 12.8%. Proinsulin was measured with a two-site enzyme linked immunosorbent assay (DAKO Ltd., Denmark AS DK-2600 Glostrup) without cross-reactivity for insulin. The reference interval provided by the manufacturer in healthy individuals was 2 to 6 pmol/L, in type 2 diabetic patients 10-15 pmol/L. CgA levels were measured by an ELISA kit (DAKO, Demnark A/S DK-2600 Glostrup), a double antibody sandwich assay. Detection limit, 2 U/l. NSE levels were determined by an EIA (Can AG Diagnostics AB, SE-41455, Gothenburg). PP levels were measured by a RIA (Euro-Diagnosticca AB SE-20211 Malmö), detection limit 3 pmol/l.

Statistical analysis

Assuming non-gaussian distribution the Mann-Whitney-U test was used to compare baseline biomarker levels between patients with insulinoma and healthy patients. Biomarker levels at baseline and after stimulation were compared using Wilcoxon matched pairs test. A two-sided value of $p < 0.05$ was considered significant. Statistical analysis was done using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, California).

Results

Patients

19 patients (14 women, 5 men) with an insulinoma were included, median age was 62 (range 33-84) years and body mass index 24.7 (19.5-34.2) kg/m^2. Localization of the insulinoma with ASVS was accurate in all 19 patients included in this study, i.e. insulin concentrations in the hepatic vein increased to 2-fold or higher of the baseline value following calcium injection into the artery supplying the tumor. Mean age of 6 patients without an insulinoma was 52 (range 25-71) years and body mass index 24.3 (20.7-30) kg/m^2.

The results of ASVS are shown in the Table and Figure 1 & 2. Data are presented as median and range. The gradients of the β-cell peptides, CgA, NSE, and PP are presented as multipliers of the baseline values

Insulin, C-peptide, and proinsulin concentrations

Median insulin concentrations by RIA in the hepatic vein were 198 (84-1242) pmol/L at baseline and 242 (72-765) pmol/L following calcium injection into the control artery (p=0.559). Insulin gradients following calcium injection into the control artery ranged from 0.6 to 1.5-fold (median 1.1) indicating absence of pathological β-cells in the region of the pancreas supplied by the control artery. Following calcium injection into the artery supplying the tumor, insulin concentrations in the hepatic vein measured by RIA increased from 223 (102-893) pmol/L at baseline to 3540 (411-14509) pmol/L (p<0.0001). Insulin gradients ranged from 2.3- to 117-fold (median 15.2); the increase in insulin concentrations was more than 2-fold in all patients with an insulinoma. Baseline levels of insulin obtained after injecting calcium into the control as well as the artery supplying the tumor were both

significantly higher compared to the levels obtained in healthy controls (p=0.001 and p=0.006, respectively).

No increase in C-peptide concentrations in the hepatic vein was observed following calcium injection into the control artery, i.e. C-peptide concentrations were 1520 (580-4640) pmol/L at baseline and 1540 (590-4420) pmol/L after calcium stimulation (p=0.609). Following calcium injection into the artery supplying the tumor, C-peptide concentrations increased from 1330 (540-4430) pmol/L at baseline to 5000 (1940-28500) pmol/L (p<0.0001), i.e. 1.7- to 32.4-fold (median 3.8). In healthy individuals, C-peptide concentrations did not increase following calcium stimulation, the gradients ranged from 0.8 to 1.6 (median 1.1) (p=0.710). C-peptide concentrations in the control arteries and tumor arteries at baseline tend to be higher compared to the healthy controls (p=0.064 and p=0.057, respectively).

Proinsulin concentrations in the hepatic vein were 37 (5-416) pmol/L at baseline and 41 (7-320) pmol/L after the calcium injection into the control artery (p=0.925). Following stimulation of the artery supplying the tumor, proinsulin concentrations increased 1.9-fold (0.7-5.3), from 34 (7-326) pmol/L at baseline to 50.6 (15-972) pmol/L, p<0.001). Baseline proinsulin levels in patients with insulinoma were higher compared to the levels obtained in healthy controls (p= 0.0001).

CgA, NSE, and PP concentrations

CgA levels in the right hepatic vein when stimulating the control artery were 14.5 (9.1-87.1) at baseline and 15.3 (7.7-89.5) U/L after the stimulation (p=0.066), the median increase was 1.2- fold (range 0.7-2.2). Injecting the tumor artery, CgA levels were 15.0 (7.2-101.3) at baseline and 13.2 (8.6-82.1) U/L after calcium stimulation (p=0.427); the gradients were 0.6 to 1.6-fold (median 1.1), i.e. no significant differences were found. CgA levels in patients

with an insulinoma (stimulation of control/tumor arteries) were significantly higher than the levels measured in healthy individuals (p=0.003 and 0.013).

Baseline NSE levels did not differ significantly between any group. NSE levelsat in the right heptatic vein were 2.5 (0.5-5.6) pmol/L after catheterization of the control artery, 3.0 (1.4-12.8) pmol/L after catheterization of the tumor artery, and 3.2 (0.9-10.6) pmol/L in healthy controls. In each group there was no significant change of the NSE level after calcium stimulation. The corresponding median gradients of NSE concentrations were 1.0 (0.1-2.3) for the control artery, 0.7 (0.2-3.0) for the tumor artery, and 1.0 (0.2-1.8) for healthy individuals.

Following calcium stimulation in the control artery, the level of PP in the hepatic vein increased from baseline 44 (32-3524) to 79 (10.5-296) pmol/L (p=0.016). PP levels increased after calcium injection into the tumor artery from 41.2 (6.3-95) to 79.5 (10-855) pmol/L (p=0.017). In healthy controls, baseline PP levels were 23.5 (6.6-68) and 52.3 (6.3-216) pmol/L after stimulation (p=0.014). Median gradients were 2.4 (0.8-7.9) in the control artery, 1.5 (0.8-4.5) in the tumor artery and 2.0 (0.6-4.8) in the healthy controls. No significant differences between the groups were found.

Table

Insulin, C-peptide, proinsulin, chromogranin A, pancreatic polypeptide (PP) and neuron specific enolase (NSE) concentrations in the hepatic vein at baseline and following injection of calcium injection into the artery supplying the tumor and a control artery in 19 patients with an insulinoma and 6 healthy individuals. Median (range) concentrations at baseline and median peak concentrations following the calcium injection are shown with * indicating a significant increase ($p<0.01$; Wilcoxon matched pairs test). Gradients represent multipliers of the baseline concentrations. $^{+}$ indicating a significant difference between gradients after calcium injection into the tumor and control artery ($p<0.001$; Wilcoxon matched pairs test).

	Tumor artery			**Control artery**			**healthy**		
	basal	**stimulated**	**gradient**	**basal**	**stimulated**	**gradient**	**basal**	**stimulated**	**gradient**
insulin (pmol/l)	223 (102-893)	3540* (411-14509)	8.0^{+} (2.3-117)	198 (84-1242)	242 (72-765)	1.1 (0.6-1.5)	124 (34-461)	102 (65-434)	1.2 (0.7-2.1)
C-peptide (pmol/l)	1330 (540-4430)	5000* (1940-28500)	3.8^{+} (1.7-32.4)	1520 (580-4640)	1540 (590-4420)	1.1 (0.9-1.4)	560 (350-2420)	760 (410-2470)	1.1 (0.8-1.6)
Proinsulin (p/mol/l)	34.0 (6.8-325.6)	50.6 (15.1-971.7)	1.9^{+} (0.7-5.3)	37.4 (5.0-415.6)	41.0 (6.5-319.5)	1.0 (0.7-2.2)	2.6 (1.9-3.9)	3.1 (1.9-4.5)	1.1 (0.9-1.3)
CgA (U/l)	15.0 (7.2-101.3)	13.2 (8.6-82.1)	1.1 (0.6-1.6)	14.5 (9.1-87.1)	15.3 (7.7-89.5)	1.2 (0.7-2.2)	7.2 (1.5-22.6)	7.8 (1.5-17.6)	1.0 (0.3-2.5)
PP (pmol/l)	41.2 (6.3-95)	79.5* (10-855)	1.5 (0.8-4.5)	44.0 (32-3524)	79.0* (10.5-296)	2.4 (0.8-7.9)	23.5 (6.6-68)	52.25* (6.25-216)	2.0 (0.6-4.8)
NSE (pmol/l)	3.0 (1.4-12.8)	2.6 (0.4-7.1)	0.7 (0.2-3.0)	2.7 (0.3-89.2)	2.5 (0.5-5.6)	1.0 (0.1-2.3)	3.2 (0.9-10.6)	2.4 (1.0-4.0)	1.0 (0.2-1.8)

Figure 1

Concentrations in the hepatic vein at baseline (ba) and after stimulation (st) by injection of calcium injection into the artery supplying the tumor and a control artery in 19 patients with an insulinoma and 6 healthy individuals. Concentrations at baseline and peak concentrations following the calcium injection are shown with * indicating a significant increase ($p<0.01$; Wilcoxon matched pairs test).

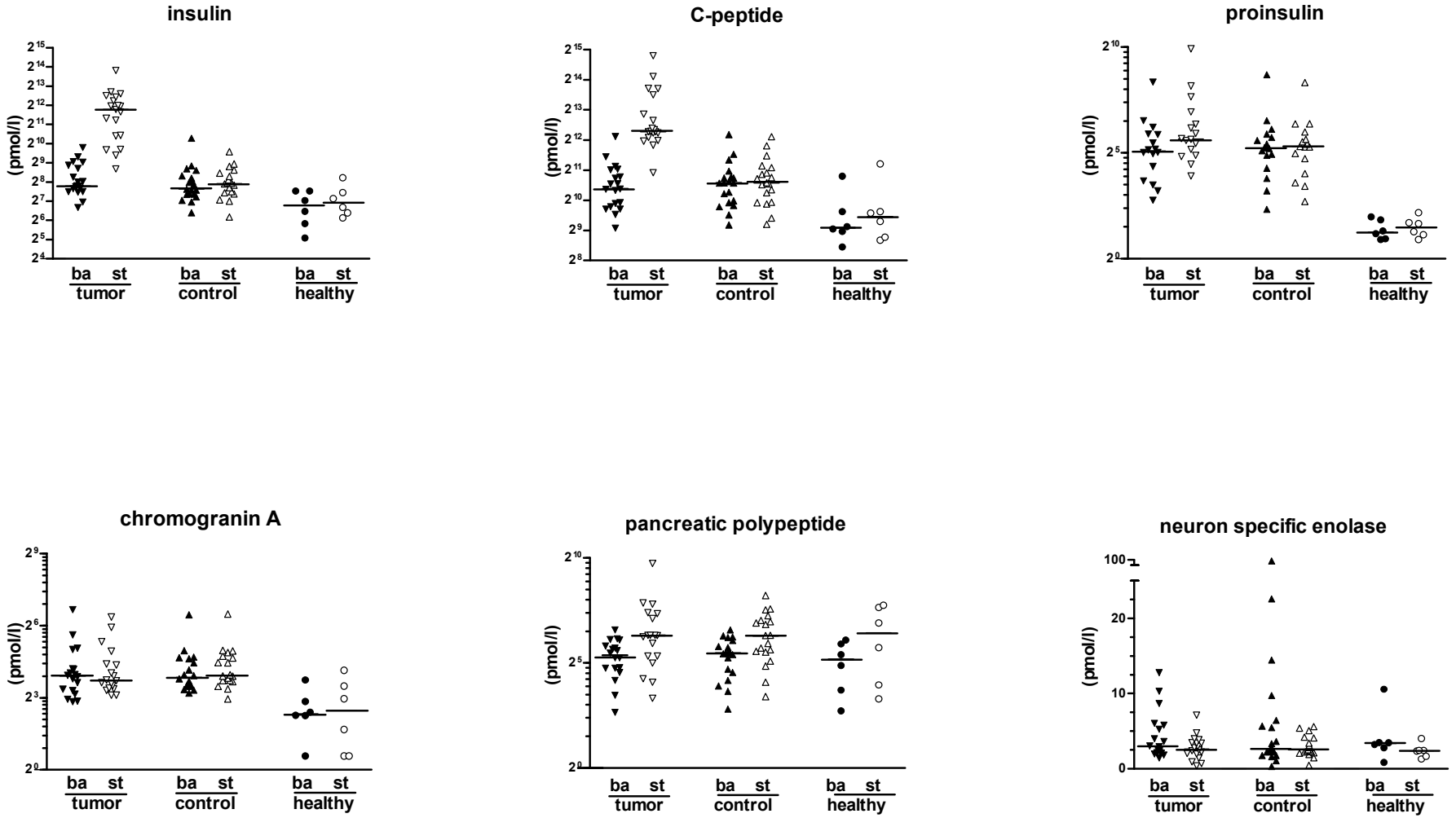
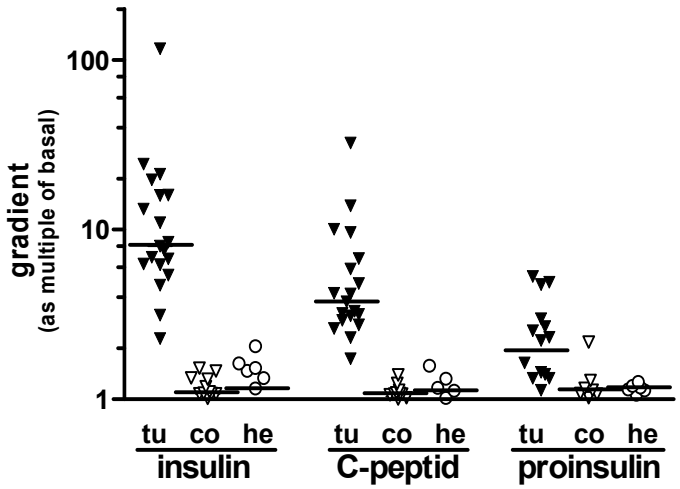


Figure 2

Gradients of insulin, C-peptide and proinsulin as multipliers of basal concentrations after stimulation by means of calcium injection into the artery supplying the tumor (tu) and a control artery (co) in 19 patients with an insulinoma and 6 healthy individuals (he).

Figure 2



Discussion

In the present study we demonstrate that insulinoma cells may be stimulated during the ASVS test by local calcium administration to release insulin, C-peptide, proinsulin, and PP. In contrast, biochemical markers of insulinoma such as CgA and NSE are not released by insulinoma cells when calcium was injected into the artery supplying the tumor.

Secretion of insulin, C-peptide and proinsulin in response to local calcium stimulation was expected. The enhanced secretion of insulin by insulinoma cells following calcium-induced depolarization of the tumor cell membrane in response to local calcium stimulation is the background of the ASVS test and represents the criterion to localize the source of excessive insulin. In the present study, insulin concentrations (determined by a traditional RIA) increased more than 2-fold in all patients with an insulinoma after calcium injection into the tumor artery. After calcium stimulation of the control artery, the maximum gradient in insulin was 1.5-fold. The more than 2-fold increase in insulin concentrations indicating the localization of an insulinoma is in agreement with the literature and the recommendation of Doppman and co-workers.[4] However, in healthy individuals the maximum gradient in insulin concentration observed in our data was 2.1-fold. In patients with insulinoma, healthy β-cells in the pancreas may be suppressed to some extent so that local calcium administration may work as a secretagogue even to a lesser extent than in β-cells of healthy individuals. These figures indicate that recommended cut-offs to localize the insulinoma during the ASVS are arbitrary and additional information to improve the accuracy of the ASVS test could be helpful at least in some patients.

We reported earlier that insulin determination by a specific insulin assay yielded significantly higher insulin gradients during ASVS than obtained by a traditional RIA; following calcium injection into the tumor as well as the control artery.[9] Thus, the traditional criterion of a more than 2-fold increase in insulin concentrations to localize an insulinoma should not be applied when insulin is measured by specific insulin immunoassays. When insulin is measured by a more specific assay, we recommend a 5- to 6-fold increase in insulin concentrations to localize an insulinoma. The introduction of new specific insulin assays with new cut-off values underlines the need for additional information to preserve the accuracy of the ASVS test.

As reported earlier, the release of proinsulin by insulinoma cells following calcium stimulation varies considerably.[14] The large range of the proinsulin gradients after calcium stimulation is explained by the well-recognized variability of insulinoma regarding the content of secretory granules for proinsulin. Gradients of proinsulin concentrations after calcium injection into the control and tumor arteries overlap and therefore, the diagnostic accuracy of the ASVS can not be improved by the determination of proinsulin concentrations. In the present study, we determined C-peptide concentrations during the ASVS test. To the best of our knowledge, this has been reported only in small series before.[15] In contrast to proinsulin, the determination of C-peptide during the ASVS test may improve diagnostic accuracy. C-peptide gradients following calcium stimulation into the control artery remained below 1.4 in all patients. In contrast, the lowest gradient after stimulation of the tumor artery in patients with an insulinoma was 1.7-fold. Therefore, we recommend a gradient of more than 1.5 for C-peptide to localize an insulinoma during the ASVS test. The maximum C-peptide gradient in healthy individuals was 1.6-fold; i.e. higher than our recommended diagnostic cut-off for insulinoma (see above). We believe that the determination of C-peptide (in addition to insulin) may help to improve diagnostic accuracy of the ASVS test. Insulin

gradients during the ASVS test depend on the methodology used to determine insulin and conflicting results may occur (i.e. higher gradients following calcium stimulation in arteries not supplying the tumor). The determination of C-peptide may be helpful in such patients. The same has been reported when comparing the utility of assessing C-peptide vs. insulin at the end of the fast. However insulin has the advantage of a shorter half-life so that recirculation and subsequently increased baseline levels is less a problem. An additional argument to determine C-peptide during the ASVS is potential hemolytic blood samples obtained during the test. Insulin concentrations may be falsely low in hemolytic samples whereas the results of C-peptide are more reliable in such circumstances. Therefore, we recommend to determine both, insulin and C-peptide during the ASVS test.

A limitation of our study is the definition of the control and tumor arteries according to the insulin gradients found. A pathological increase in insulin concentration following calcium injection into a control artery may occur when calcium stimulates the insulinoma by recirculation or collateral vessels. To address this limitation we have included six patients undergoing the ASVS test during celiac angiography of the pancreas without evidence for an insulin-secreting tumor.

Additionally, we tested, whether the accuracy of the ASVS test could be improved by the determination of additional peptides. However, our data show that neither the determination of CgA, NSE nor PP is helpful to improve the diagnostic accuracy of the ASVS test. We show that CgA and NSE are not released by insulinoma cells when calcium in injected into the artery supplying the tumor. CgA is a glycoprotein which is found in the secretory granules of most neuroendocrine cells,[16] therefore, it might be considered as surprise that CgA is not released by insulinoma cells during the ASVS test, when the content of secretory granules is released. However, granins tend to bind calcium with low affinity but high capacity and then

aggregate in vitro at low pH in the presence of calcium.[17-20] These aggregation characteristics may be responsible that CgA is not released following local calcium administration, neither in patients with an insulinoma nor in healthy individuals. Neuron-specific enolase, the glycolytic isoenzyme of the enolase gamma-gamma dimer, is a specific marker of the neuroendocrine system and derivative of neuroendocrine tumors.[21, 22] Our data suggest that NSE is not be released by local calcium administration, neither in patients with an insulinoma nor in healthy individuals. The secretion of PP was associated with a depolarization-dependent elevation of the intracellular calicum concentration.[23] The determination of PP during the ASVS indicated that PP may be released by healthy islet cells as well as insulinoma cells. Thus, the determination of PP is not helpful to improve diagnostic accuracy of the ASVS test.

In conclusion, the determination of C-peptide, in addition to insulin, during the ASVS test may help to improve diagnostic accuracy. We recommend a more than 1.5-fold increase in C-peptide following calcium stimulation to localize an insulinoma. The determination of CgA, NSE and PP does not improve diagnostic accuracy of the ASVS test.

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